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A search for association between hereditary hemochromatosis HFE gene mutations and type 2 diabetes mellitus in a Polish population

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Maciej T. Małecki[ABCDEFG], **Tomasz Klupa**[B], **Małgorzata Waluś**[B], **Wojciech Czogała**[B], **Paul Greenlaw**[B], **Jacek Sieradzki**[B]

Department of Metabolic Diseases, Medical College, Jagiellonian University, Krakow, Poland

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Summary

Background: Hereditary hemochromatosis (HH) is characterized by excess iron deposition. Two mutations in the HFE gene are associated with HH. Heterozygous carriers of HFE mutations are at higher risk of developing type 2 diabetes mellitus (T2DM). The aims of our project were to identify the frequency of C282Y and H63D mutations in a population from the Małopolska region of south-eastern Poland, and to search for an association of HFE mutations with T2DM.

Material/Methods: We included 391 individuals in this study: 222 T2DM patients and 169 controls. Genotypes were determined by electrophoresis of the DNA digestion products from SnaBI and DpnII, respectively. Differences in distributions between the groups were then analyzed by the chi-squared test.

Results: The frequency of wild/C282Y alleles was 98.2%/1.8% in T2DM patients and 96.7%/3.2% in controls (p=0.19). The frequency of wild/H63D alleles was 85.6%/14.4% and 88.8%/11.2% (p=0.19), respectively. The distribution of genotypes was not statistically different. However, in stratified analyses based on age of T2DM onset and gender, we observed a higher prevalence of wild/H63D and H63D/H63D genotypes among T2DM patients diagnosed at ⩾ 49 years of age, the mean age for the entire group (p=0.018), and among male T2DM individuals (p=0.005) than in controls.

Conclusion: The frequency of HH-associated mutations in this population from south-eastern Poland is similar to other Caucasians. We found no evidence for the association of the C282Y mutation with T2DM. The results do suggest, however, that the H63D mutation may play a role in the pathogenesis of late onset T2DM and in males in this Polish population.

key words: **HFE gene • hemochromatosis • Type 2 diabetes mellitus**

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Author's address: Maciej T. Malecki, MD PhD, Department of Metabolic Diseases, Jagiellonian University, Medical College, 15 Kopernika Street, 31-501 Krakow, Poland, email: mmalecki@cm-uj.krakow.pl, malecki_malecki@yahoo.com

BACKGROUND

Type 2 diabetes mellitus (T2DM) is a very common chronic disease that affects almost 200 million people worldwide [1]. It is characterized by impaired insulin secretion and alterations in peripheral insulin action. The phenotype of T2DM is created by an interaction of both genetic and environmental factors [2]. Several genes have been associated with rare monogenic forms of T2DM [3–6]. These monogenic forms of T2DM are usually characterized by impaired insulin secretion [3,4,5], with the exception of insulin receptor mutations that result in extreme insulin resistance [6]. Recently, some evidence has been found which appears to indicate the association of calpain 10 and peroxisome proliferator-activated receptor (PPAR)-γ gene polymorphisms [7,8] with the more common, complex T2DM forms that are accompanied by both insulin resistance and abnormalities in insulin secretion. For the most part, however, the molecular pathophysiology of T2DM is not yet fully understood. One of the most interesting candidates for complex forms of T2DM is the HFE hereditary hemochromatosis (HH) gene [9]. HH is a frequent, autosomal recessive metabolic disorder characterized by excess iron deposition in various organs. This gene is related to the major histocompatibility complex class I family [10,11]; its protein binds to the transferrin receptor, and in so doing reduces its affinity for iron-loaded transferrin 5 to 10 times [12]. Recently, two mutations in the HFE gene, one a substitution of tyrosine for cysteine at codon 282 (C282Y) and the second a substitution of histidine with aspartate at codon 63 (H63D), have been linked to HH [9]. The C282Y mutation affects the HFE protein's structure and function, disrupting its ability to transport and present on the cell's surface [11]. The H63D mutation seems to have a less prominent biological impact, but acts synergistically with the C282Y mutation [11]. The identification of HFE as a gene responsible for hemochromatosis has been confirmed through the development of an animal model [13]. It is well known that T2DM and HH diagnoses freqquently coexist [14,15]. Therefore, it would seem a reasonable hypothesis that not only homozygous carriers of HFE mutations, but also heterozygous carriers as well, may be at higher risk of developing glucose metabolic abnormalities. This theory is further supported by the fact that subtle biochemical defects can often be seen in the heterozygous state. For example, higher serum ferritin levels and transferrin saturation have been observed [16].

The studies performed thus far have not provided a clear answer regarding a possible association of the HFE gene with T2DM, and substantial differences have been shown between ethnic groups [10]. Thus it is important to determine the possible role of this gene in various populations.

The aims of our study were:

1) To identify the frequency of C282Y and H63D mutations in a population from the Małopolska region of south-eastern Poland, and
2) To search for an association between HFE mutations and T2DM in this population.

MATERIAL AND METHODS

We included 391 unrelated individuals in this study: 222 T2DM patients and 169 non-diabetic controls. All study participants were Caucasians and residents of the Malopolska region of south-eastern Poland. WHO definitions and criteria were used during patient recruitment [17]. Patients received a standard questionnaire that contained questions regarding their age at T2DM diagnosis, family history, treatment method, and other medical issues. Only patients with a clinical diagnosis of T2DM, age of onset later than 35 years, and no insulin therapy for at least the two years immediately after diagnosis were recruited. The control group consisted of individuals with normal fasting glucose and a negative family history of T2DM among all first-degree relatives. This group primarily constituted spouses of T2DM patients and volunteers from the Department of Metabolic Diseases. All participants underwent a basic physical examination, which included the measurement of height, weight, and blood pressure. The fasting glucose level was determined by the enzymatic method. HbA_{1c} was measured in the T2DM patient group by the HLPC method (Biorad). This study was performed in compliance with the Helsinki Declaration and approved by the Ethical Committee of the Jagiellonian University's College of Medicine.

DNA from the examined individuals was isolated from peripheral blood lymphocytes using GIBCO's DNAzol Reagent [18]. This DNA was then used to amplify the HFE gene fragments containing the described variants. We used the primer sequences published by Jazwinska et al. for the C282Y mutation [19], with later modification of the reverse primer sequence, as described by Jeffrey et al, to assure specificity of genotyping [20]. Previously published primers were also used for the H63D mutation [9]. The PCR products were digested according to the supplier's recommendation (Fermentas) using specific restriction enzymes, SnaBI and DpnII, to detect mutations at residues 282 and 63, respectively. The C282Y mutation creates an SnaBI restriction site, while the H63D mutation abolishes a site for DpnII. Digestion products were separated on an ethidium-bromide stained, 3% agarose gel. The results were documented digitally and stored as computer files using Biocapt software (Vilbert-Lourmant).

Statistical analysis

Differences in the distribution of alleles and genotypes were assessed using the chi-squared test. Hardy-Weinberg equilibrium was examined with the goodness-of-fit chi-squared test. Comparisons between study groups were made with Student's t-test for quantitative traits. Qualitative traits were analyzed using the chi-squared test. For all statistical analyses, STATISTICA for Windows was used (Statsoft Inc, Tulsa, Oklahoma, USA).

RESULTS

The amino acid variants of the HFE gene at residues 63 and 282 were determined in 391 individuals: 222 T2DM patients and 169 non-diabetic controls. Clinical characteristics for both study groups can be found in Table 1. The genotypes were in Hardy-Weinberg equilibrium for both cases and controls. Details of allele and genotype distributions are shown in Table 2. In general, there was no difference between the two groups, but a trend toward higher frequency of wild/H63D and H63D/H63D genotypes was observed among T2DM cases. There was one C282Y homozygote and one compound heterozygote in each group. In addition to the results shown in the Table 2, we performed two stratified analyses. For this analysis, we divided our patient group based on age of onset (<49 or ⩾49 years, the mean age of T2DM onset for the entire patient group) and gender. In T2DM cases who developed disease later in life (114 individuals), the frequency of the H63D allele was 16.7%, while wild/H63D and H63D/H63D genotypes constituted 32.4% in this subgroup. The prevalence of the wild/H63D and H63D/H63D genotypes among these T2DM patients was significantly different from controls (1 d.f., χ^2=5.51; p=0.018). Similarly, male patients (108 individuals) with T2DM were characterized by a higher frequency of alleles and genotypes with aspartate (18.1% and 35.2%, respectively). These values were also significantly different from the control group (1 df, χ^2=5.11; p=0.023 and 1 df, χ^2=7.7; p=0.005 for alleles and genotypes, respectively). Additionally, we looked at the clinical characteristics of the H63D allele carriers at residue 63 of the HFE gene. Overall, there were 61 hetero- or homozygous carriers of this variant. On average, these patients were 50.9±9.0 years of age at diagnosis, while the remaining members of the patient group developed T2DM at age 48.9±7.9 (p=0.12). There were significantly more men in this group than among diabetic non-carriers of the H63D allele (62.3% vs. 43.5%, p=0.012). BMI among carriers was 30.3±4.6, very similar to members of the non-carrier T2DM group (30.4±5.9). The allele and genotype distributions observed in men and women from the control group for H63D were similar. The results from stratified analyses for the mutation at residue 282 were not significant.

Table 1. Characteristics of the study groups.

Parameters	T2DM	Controls
Sex (women/men)	114/108	95/74
Age at examination (years)*	59.8±8.9	55.1±14.7
Age at diagnosis (years)*	49.1±7.7	N/A**
Duration of the disease (years)*	10.7±6.9	N/A
BMI (kg/m²)*	30.3±5.6	27.5±5.8
Fasting glucose (mmol/l)*	9.2±9.0	4.8±0.8
$HbA_{1c\ (\%)}$*	7.8±1.8	N/A**
% on insulin treatment	55.0%	N/A**
% with positive family history of T2DM in first degree relatives	46.4%	0.0%

The high proportion of insulin-treated type 2 diabetes patients was related to the relatively long mean duration of diabetes. Fasting glucose was measured in T2DM patients prior to administration of morning dose of antidiabetic medications.

*Data are given as mean±SD; **N/A = not applicable

DISCUSSION

In this case-control study we searched for an association of two mutations in the HFE gene with T2DM in a homogenous, Caucasian population from the Małopolska region of south-eastern Poland.

The studies published to date have not provided an unambiguous answer regarding the role of HFE mutations in the pathogenesis of T2DM. Four studies performed in different Cauciasian populations from Germany, the UK, France, and New Zealand were not able to show an association of either mutation with T2DM [21–24]. However, three other studies suggested that indeed this gene might influence susceptibility to T2DM. For example, in one of these studies, involving a Spanish population, it was observed that the H63D allele's frequency at residue 63 of the HFE gene was significantly increased in T2DM patients [22]. In two other Caucasian studies, the C282Y allele at residue 282 was

Table 2. Allele and genotype distribution of the HFE gene in T2DM patients and controls.

	Allele			Genotype		
Codon 63	wt	H63D	χ^2=1.7 1 .d.f p=0.19	wt/wt	wt/H63D and H63D/H63D	χ^2=2.8 1.d.f p=0.09
T2DM patients	380 (85.6 %)	64 (14.4 %)		161 (72.5%)	61 (27.5 %)	
Controls	300 (88.8%)	38 (11.2%)		135 (79.9%)	34 (20.1%)	
Codon 282	wt	C282Y	χ2= 1.7 1.d.f p=0.19	wt/wt	wt/C282Y and C282Y/C282Y	χ2= 1.7 1.d.f p=0.18
T2DM patients	436 (98.2%)	8 (1.8%)		215 (96.8%)	7 (3.2%)	
Controls	327 (96.8%)	11 (3.2%)		159 (94.1%)	10 (5.9%)	

wt –wild type

more prevalent in the T2DM patient groups [23,24]. Interestingly, one of these studies came from a Polish population originating from the Silesia region [24]. Moczulski et al. have also suggested a possible role of the H63D allele at residue 63 of the examined HFE gene in T2DM susceptibility. This prompted us to test this observation in a population from the neighboring Małopolska region. In the present study, we were not able to confirm the previously reported role of the C282Y mutation in the pathogenesis of T2DM in a Polish population. In fact, we observed the opposite trend, towards a higher frequency of this mutation in controls than in T2DM cases. We did observe, however, a tendency toward higher prevalence of the H63D allele in the group of T2DM patients. Additionally, we performed stratified analyses of the data, hypothesizing that, since HH usually develops during the later stages of adulthood [10,15], the effects of heterozygous mutations may potentially be detectable in patients with late onset T2DM. Stratification was further justified considering that, since iron loss is often associated with menses and pregnancy, the clinical expression of HFE gene variants would presumably be more severe in men than in women [28]. Indeed, we found evidence supporting the notion that the H63D mutation may constitute a susceptibility allele for late onset forms of T2DM and in males. While it has been shown that the H63D mutation does not have such profound effects on the HFE protein's function as the C282Y mutation, it does reduce the HFE protein's functional influence on the transferrin receptor [11]. In addition, patients with T2DM who were heterozygous for the H63D allele have been found to exhibit some physiological abnormalities, such as high plasma ferritin level [25]. Thus an iron overload that leads to decreased β-cell secretion of insulin and to insulin resistance [29,30] may be one of the mechanisms of T2DM pathogenesis in these individuals. However, there is a possibility that another, as yet unidentified mechanism not associated with iron metabolism exists through which the H63D mutation of the HFE gene influences susceptibility to T2DM

Additionally, the difference in terms of the C282Y mutation observed between two studies using Polish ethnic population warrants further discussion. While both projects used a case-control study design, a model that is sensitive and optimal in the search for association between a polymorphism and a complex disease [31], the two studies produced different results in respect to this mutation. While an association between T2DM and the tyrosine variant at residue 282 was found in the Silesia population, the opposite trend was observed in the study group from the Malopolska region. One possible explanation for this discrepancy is that the results from the Silesia study constitute a type 1 error. This error could have occurred because of population stratification [32,33]. It is well known that case-control analyses may identify spurious associations if case and control subjects are drawn differentially from two or more subpopulations, when marker allele frequencies and disease prevalence differ across these subpopulations. As the population from the Silesia region experienced several waves of migration, particularly in the 20th century, and subsequently ethnic admixtures, this possibility cannot be entirely excluded. It should also be emphasized that, with an observed minor allele frequency of 1–2%, the C282Y mutation represents a relatively poor marker for examination in complex diseases, such as T2DM [33]. This mutation may also be prone to producing spurious associations as a result of random variation. These types of inconsistencies have been previously described, not only between different races and nations, but also within the same ethnic groups [7,34,35,36]. These differences may have occurred not only due to population stratification and random variation, as mentioned above, but also as a result of power differences that are influenced by the size of the study groups, as well as allele and disease frequency [33]. We postulate that to fully determine whether H63D and C282Y alleles are susceptibility variants for T2DM, additional studies in different populations are required. It seems likely that, as was the case for the PPARγ Pro12Ala variant, a large meta-analysis could eventually help to further define the role of the examined sequence differences [8].

CONCLUSIONS

The frequency of HH-associated mutations in a population from the Małopolska region of south-eastern Poland is similar to that found in other Caucasian populations. We found no evidence supporting the recently reported association of the C282Y mutation with T2DM in this Polish population. The results of our study do, however, suggest that the H63D mutation may play a role in the pathogenesis of late onset T2DM and in males in our population.

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